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Bacteriostatic effect of human milk on *Escherichia coli*: the role of IgA

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Summary. Previous work showed that lactoferrin was involved in the bacteriostatic effect of human milk on *E. coli* O111. Further experiments on the general nature of this effect have been carried out together with an examination of the role of IgA. Milk samples from different individuals differed in their ability to produce bacteriostasis of three pathogenic serotypes of *E. coli*. The bacteriostatic effect was stable to heating at 60° for 35 min. As in the case of serum, the bacterial iron transporting compound, enterochelin, abolished the bacteriostatic effect of human milk. IgA was isolated from the milk samples in two forms which appeared to differ in molecular weight. When mixed with lactoferrin, some of these fractions induced bacteriostasis which could be reversed by Fe^{3+}. Since the fractions were devoid of bactericidal activity in the presence of complement it appeared that IgA was involved in the induction of bacteriostasis. It was also concluded that the mechanism of bacteriostasis was identical in serum and milk. These results are discussed in relation to both the protective effect of feeding colostrum and milk and also the resistance of the adult gastrointestinal tract to infection.

INTRODUCTION

Breast-fed infants suffer less from gastrointestinal disorders, upper respiratory tract infections and otitis media than do wholly bottle fed infants (Gerrard, 1974). Conversely, the weaning of infants in a heavily contaminated environment leads to the onset of a variety of infections (Mata & Urrutia, 1971). The most convincing demonstration of the protective power of human milk was the stopping of two long standing outbreaks of gastroenteritis caused by antibiotic resistant *E. coli* O111 (Svirsky-Gross, 1958; Tassovatz & Kotsitch, 1961). In piglets, Kohler (1974) showed that the feeding of immune colostrum produced a 10,000 fold reduction in the population of *E. coli* O149 in the small intestine compared to that of the controls receiving normal colostrum. The results of routine bacteriological studies showed that the faeces of breast-fed infants contain fewer serotypes of *E. coli*, including those carrying the K1 antigen involved in neonatal meningitis (Sarff, McCracken, Schiffer, Glode & Robbins, 1975) than do those of bottle fed infants (Orskov & Biering Sorensen, 1975). Whilst the feeding of breast milk favours the development of a large population of lactobacilli in the large intestine (Bullen & Willis, 1971), there is also evidence that the population of coliforms may be inversely related to the antibody content of the colostrum and milk (Michael, Ringenback & Hottenstein, 1971).

Three major factors have been proposed to account for the protective effect of milk in the small intestine. First, the ability to neutralize bacterial enterotoxins (Stoliar, Pelley, Kaniecici-Green, Klaus & Carpenter, 1976). Second, the prevention of the

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adhesion of the bacteria to the mucosal surface of the small intestine (Fubara & Freter, 1973). Similar antigens responsible for the adhesion of human pathogenic serotypes of *E. coli* have been described recently (Evans, Silver, Evans, Chase & Gorbach, 1975). Third, the ability of colostrum, milk and serum to inhibit the multiplication of *E. coli* in the small intestine was clearly demonstrated in pigs (Kohler, 1974; Smith, 1972). In the case of the guinea-pig, it was suggested that the suppression of growth was due to the combined effect of the partially saturated iron binding protein, lactoferrin and antibody (Bullen, Rogers & Leigh, 1972). In this paper, further aspects of the general nature of the bacteriostatic effect of human milk are presented together with an examination of the role of IgA.

MATERIALS AND METHODS

Bacteria

E. coli O111/K58/H2 was as used in the previous work (Bullen *et al.*, 1972). *E. coli* O1/K1/H was obtained from Dr I. Orskov, Statens Seruminstitut, Copenhagen, Denmark, whilst *E. coli* O55/K59/H- was obtained from Dr B. Rowe, Central Public Health Laboratory, Colindale. Organisms from a 3-h broth culture were collected by centrifugation and suspended in 10% (v/v) papain digest broth in saline and the population was then estimated by nephelometry with the aid of a standard curve. Viable counts were made by spreading 0·10 ml volumes of appropriate dilutions on blood agar plates.

Human milk

Normal human milk was obtained from Dr J. M. Levi, Paediatric Department, University College Hospital, London, and Dr F. E. Hytten, Clinical Research Centre, Harrow. The samples, designated A, B, C and D, were stored at −20°. Milk from mothers receiving antibiotics was excluded from this study.

Human lactoferrin

Lactoferrin was isolated from human milk as previously described (Bullen *et al.*, 1972) but the concentrate from only 100 ml milk was used as the starting material. The lactoferrin was finally purified by gel filtration on a 3·2 × 95 cm column of Bio gel A-5 M (200–400 mesh) in phosphate buffered saline pH 7·4. The concentrated solution, after dialysis against 0·15 N NaCl containing 0·02% $NaHCO_3$, had a total iron binding capacity of 0·385 mM and was 17·4% saturated with Fe^{3+} (Rogers, 1976). On immunodiffusion, it failed to react with anti-human IgM, IgA or IgG.

Enterochelin

This bacterial iron transporting compound was isolated from culture fluids of *E. coli* K12 as described by Rogers, Synge, Kimber & Bayley (1977).

Boivin antigen

This material was isolated from *E. coli* O111 by S. P. Fitzgerald, using the method of Staub (1965).

Sera

Foetal calf serum was obtained in the frozen state from Wellcome Reagents Ltd. and stored at −70°. Its ability to act as a source of complement in the bactericidal test was checked periodically using horse antiserum to *E. coli* O111 (Rogers, 1976).

Immunoelectrophoresis and immunodiffusion

Microimmunoelectrophoresis and immunodiffusion were carried out in 1% agarose gel in 0·03 ionic strength barbital acetate buffer pH 8·3. Precipitin lines were developed with specific antiserum to human IgM, IgA and IgG (Wellcome Reagents Ltd.).

Bactericidal test

The test is based on that of Rowley (1968). Foetal calf serum, 1·0 ml, was mixed with the test sample, up to 0·5 ml, and 0·02% $MgCl_2$ in PBS was added to give a total volume of 2·0 ml. 0·05 ml of a suspension containing 2×10^4 *E. coli* O111 was then added and the samples were incubated for 2 h at 37° after which viable counts were made.

Bacterial culture medium

This medium was used to examine the antibacterial effects of the protein fractions from human milk. Bovine casein, 1·0 g, was stirred for 30 min with 100 ml 0·85% NaCl maintained at pH 11·5 by careful addition to 1 N NaOH. The pH was then lowered to 7·4 by the addition of 1 N HCl. The supernatant was decanted from the residue and clarified by filtration through fluted filter paper. The filtrate was made 10% in lactose, 0·2% in glucose and 0·2%

in bovine serum albumin (fraction V). Sufficient 10 times concentrated medium 199 (without antibiotics, phenol red and $NaHCO_3$) was then added to give one half normal strength medium (Bullen *et al.*, 1972) and the pH was raised to 8·1 by the careful addition of 1 N NaOH. The medium was sterilized by filtration through a 0·45 μm filter and stored at 4°.

Bacterial growth in human milk and milk fractions

Samples of whole milk, 3·0 ml, containing 0·6% $NaHCO_3$ were stirred by means of magnetic followers in jacketed culture vessels at 37° under a gas mixture containing 10% O_2–5% CO_2–85% N_2 flowing at 50 ml per minute (Rogers, 1973). The fractions were tested in a total volume of 3·0 ml consisting of 1·2 ml of the medium described above, 0·3% $NaHCO_3$ and saline. When required, lactoferrin was added to give a total iron binding capacity of 20 μM. Samples for viable counting were diluted 1:10 in broth-saline and homogenized for 1 min in an ice-bath (MSE microhomogenizer).

Gel filtration

Gel filtration was carried out on 3·2 × 95 cm columns of Bio gel A-5 M and A-0·5 M, 200–400 mesh (Bio Rad Laboratories) by pumping the columns upwards at 15–20 ml/h with phosphate buffered saline ionic strength 0·15, pH 7·4 at 4°.

Ion exchange chromatography on diethylaminoethyl cellulose

A 2·0 × 50 cm column of DEAE-cellulose (Whatman DE 52, microgranular) was used at 4°.

Concentration of fractions

Fractions were concentrated in a Sartorius high pressure filter no. 16208 fitted with a membrane no. 12136. The volume of each sample was finally adjusted to 3·0 ml with PBS pH 7·4 and the protein content estimated from the optical density at 280 nm using $E^{1\%}_{1cm} = 13{\cdot}9$ for human IgA (Tomasi & Bienenstock, 1968).

RESULTS

Bacteriostatic effect of whole milk

The bacteriostatic effect was examined in the presence of bicarbonate buffer at pH 7·4–7·5 (Bullen *et al.*, 1972). Milk sample A exerted a bacteriostatic effect on *E. coli* O111 but the specificity was not examined further. The bacteriostatic effect of milk C against *E. coli* O1, O55 and O111 is shown in Fig. 1. Milk B was also active against *E. coli* O1 and O111 but somewhat less active against O55 (data not shown). Milk sample D was active against *E. coli* O111 but had no activity on the other serotypes (Fig. 2). Dilution of milks B and C with one quarter strength medium 199 containing sufficient lactoferrin to maintain a total iron binding capacity of at least 20 μM showed that the bacteriostatic effect against

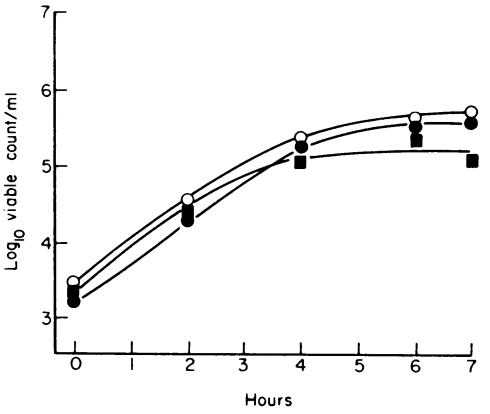

Figure 1. Bacteriostatic effect of human milk sample C on *E. coli*: (●) serotype O1; (○) serotype O55; (■) serotype O111.

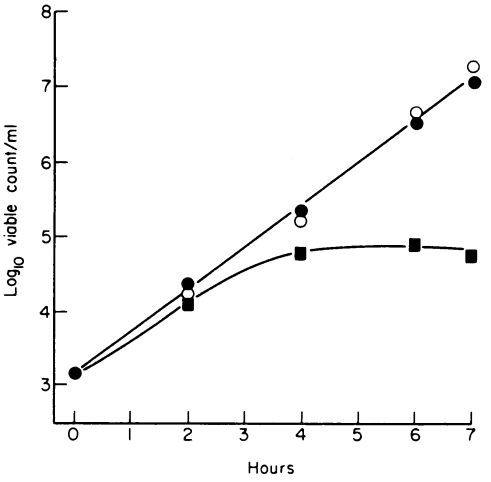

Figure 2. Growth curves of *E. coli* in human milk sample D: (●) serotype O1; (○) serotype O55; (■) serotype O111.

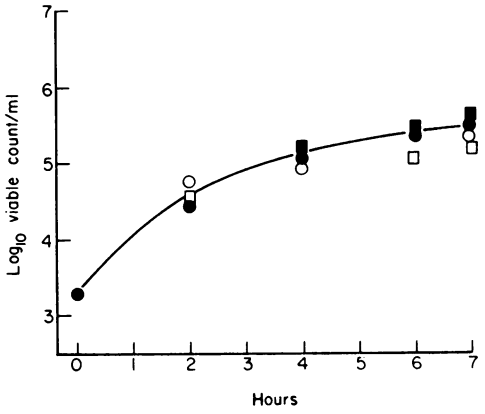

Figure 3. Effect of diluting human milk C on its bacteriostatic effect against *E. coli* O111. One quarter strength medium 199 containing lactoferrin to maintain a total iron binding capacity of 20 μM was used as diluent: (●) undiluted; (○) diluted 1 : 2; (■) diluted 1 : 4; (□) diluted 1 : 8.

E. coli O111 was undiminished at a dilution of 1:8 (Fig. 3).

Effect of pasteurization

Milk samples A and D were heated in a water bath at 60° for 35 min. Prior to this, the samples contained 3×10^2 and 3×10^4 *Staphylococcus albus* per ml respectively. After heating, no *S. albus* could be detected. The bacteriostatic effect of both samples of whole milk against *E. coli* O111 appeared to be unimpaired by this treatment (data not shown).

Bactericidal effect

The results of the tests for the bactericidal effect of 25% v/v whole milk against *E. coli* O111, using foetal calf serum as a source of complement are shown in Table 1. Clearly milks C and D both exert a marked bactericidal effect under these conditions.

Table 1. Bactericidal test of 25% (v/v) human milk samples against *E. coli* O111 in 50% (v/v) foetal calf serum

Sample	Viable counts at 2 h* (bacteria/ml)
Control, no addition	$4{\cdot}6 \times 10^5$
Sample B	$1{\cdot}4 \times 10^4$
Sample C	$6{\cdot}5 \times 10^2$
Sample D	$9{\cdot}7 \times 10^2$

* The inoculum was $1{\cdot}4 \times 10^4$ bacteria/ml.

Effect of adsorption with heat killed *E. coli* O111

Samples of milks B and C were adsorbed with 1×10^{10} heat killed *E. coli* O111 per ml for 2 h at 37° followed by 16 h at 4°. After removing the bacterial cells by centrifugation, the bacteriostatic effect of both samples against *E. coli* O111 appeared to be unimpaired (data not shown).

Effect of adsorption with Boivin antigen from *E. coli* O111

A 3 ml sample of milk B was mixed with 0·6 ml saline containing a total of 15 mg Boivin antigen (pH 7·5), incubated at 37° for 2 h and then allowed to stand for 16 h at 4°. The mixture, together with the control to which had been added 0·6 ml saline, were then made 0·6% in $NaHCO_3$ and inoculated with *E. coli* O111. Fig. 4 shows that the bacteria grew rapidly in the adsorbed milk suggesting that the antibody had reacted with the lipopolysaccharide of the bacterial cell.

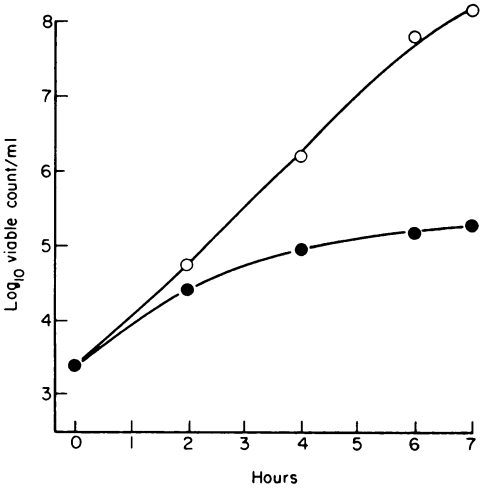

Figure 4. Effect of adsorbing human milk sample B with Boivin antigen from *E. coli* O111 (5 mg/ml) on the bacteriostatic effect against *E. coli* O111: (●) saline treated control; (○) adsorbed sample.

Effect of enterochelin on bacteriostasis

Recent work has shown that the bacteriostatic effect of serum on *E. coli* O111 can be abolished by adding the bacterial iron transporting compound, enterochelin, as late as 5 h after the beginning of the experiment (Rogers *et al.*, 1977). Similar results were obtained by adding 0·1 μM enterochelin to milk D which had been inoculated with *E. coli* O111 (Fig. 5).

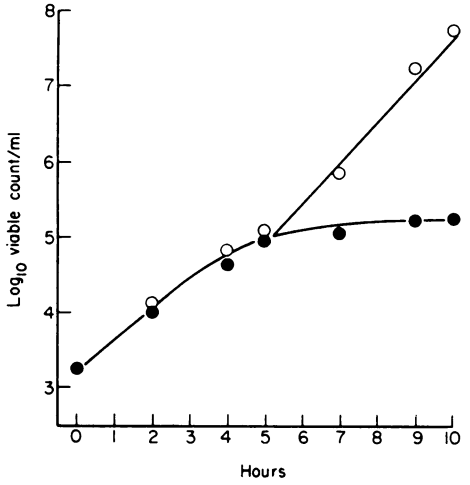

Figure 5. Effect of adding enterochelin (0·1 μM) at 5 h on the bacteriostatic effect of milk sample D against *E. coli* O111: (●) control; (○) enterochelin added.

Isolation and antibacterial effects of immunoglobulins from individual milk samples

Sample A. Three portions each consisting of 4·0 ml of 7 times concentrated, clarified milk (Bullen *et al.*, 1972) were fractionated by gel filtration on a 3·3 × 95 cm column of Bio gel A-5 M. The main IgA fraction so obtained, was purified further by ion exchange chromatography on a 3 × 50 cm column of DEAE cellulose equilibrated with 0·03 M tris HCl pH 8·0. Linear gradient elution using a limiting buffer of 0·03 M Tris HCl containing 0·3 M NaCl pH 8·0, 400 ml each, gave two main fractions; the first contained mainly IgA and a trace of IgM whilst the second which contained at least three components including IgM was not examined further. Final purification of the IgA was achieved by gel filtration on Bio gel A-5 M as described above. Unfortunately only 7 mg of IgA was recovered from 84 ml milk by this method. When tested at a concentration of 0·5 mg per ml in the presence of lactoferrin, this material exerted a bacteriostatic effect against *E. coli* O111 although lactoferrin itself had no such effect (Fig. 6).

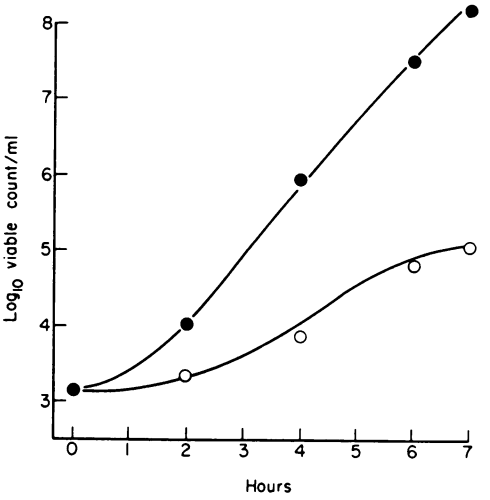

Figure 6. Effect of IgA (0·5 mg/ml) from milk sample A on *E. coli* O111: (●) medium plus lactoferrin (TIBC 20 μM); (○) medium plus lactoferrin plus IgA.

Sample B. A volume of 45 ml of clarified and 4 times concentrated milk was dialysed against 2·0 l 0·01 M sodium phosphate pH 8·0 prior to ion-exchange chromatography on a 2 × 50 cm column of DEAE cellulose equilibrated with the same buffer. Linear gradient elution to a limiting buffer of 0·01 M sodium phosphate containing 0·2 M NaCl pH 8·0, 600 ml each, was then carried out. The results obtained were similar to those of Hanson & Johansson (1962) in that six major protein fractions were obtained, immunodiffusion tests showed that IgA was present in all but the first of these. In the case of fraction II, for example, immunoelectrophoresis gave 2 lactoferrin arcs, one of which also reacted with anti human IgA. Gel filtration of fraction II on Bio gel A-5 M produced a clear separation of IgA and lactoferrin. More IgA was obtained in the same way from fraction III, this gave a total of 24·7 mg IgA. Immunodiffusion tests with a solution containing 3·3 mg per ml showed that it gave a strong reaction with anti human IgA but failed to react with anti human IgG or IgM. This suggests that the IgG and IgM

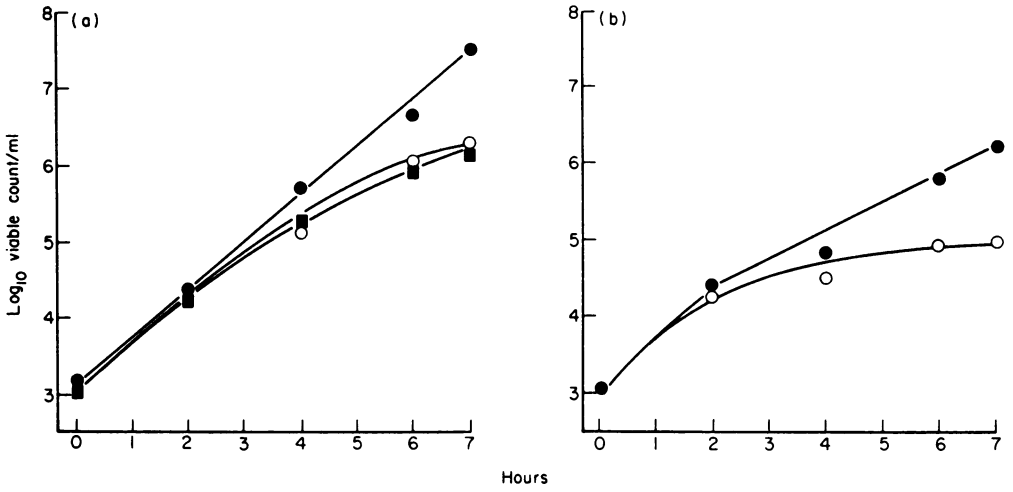

Figure 7. Effect of IgA (1·5 mg/ml) from milk sample B and lactoferrin (TIBC 20 μM) on *E. coli*:
(a) (●) serotype O1 with lactoferrin; (○) serotype O1 with lactoferrin plus IgA; (■) serotype O111 with lactoferrin plus IgA.
(b) (●) serotype O55 with lactoferrin; (○) serotype O55 with lactoferrin plus IgA.

contamination was less than 0·3% (Heddle & Rowley, 1975a). When tested at a concentration of 1·5 mg per ml in the presence of lactoferrin, the IgA reduced the bacterial population to less than 10% of that attained with lactoferrin alone (Figs 7a and b). When compared to the whole milk, however, it appears that this mixture does not represent the full antibacterial activity, particularly against serotypes O1 and O111.

Further fractionation of fraction 1 from DEAE cellulose by gel filtration on Bio gel A-5 M showed that it contained only high molecular weight material, no IgG could be detected. When examined in the bactericidal test at a concentration equivalent to that of whole milk, the high molecular weight fraction proved to be inactive.

An attempt was made to isolate an IgM containing fraction by gel filtration of 8·0 ml of clarified and 3 times concentrated milk on Bio gel A-5 M. The fraction obtained, although concentrated to 3·0 ml, failed to react with anti serum to human IgM but did react with anti human IgA. The material was inactive when tested at a dilution equivalent to the original milk, in the bactericidal test against *E. coli* O111. When mixed with lactoferrin, the fraction did not produce true bacteriostasis; rather it increased the generation time of the organism from 21 to 61 min.

Sample C. An IgA containing fraction was isolated by gel filtration of 10 ml of clarified and 2 times concentrated milk on Bio gel A-5 M. The IgA was finally purified by ion exchange chromatography in a 2 × 50 cm column of DEAE cellulose equilibrated with 0·075 M Tris HCl pH 8·0 by linear gradient elution with a limiting buffer of 0·075 M Tris HCl containing 0·5 M NaCl pH 8·0 (400 ml each). The final product, 26 mg IgA, when dissolved in 5·0 ml PBS contained no detectable protein other than IgA. When tested at a concentration of 1·5 mg per ml in the presence of lactoferrin, the growth patterns of both *E. coli* O55 and O111 were similar to those obtained in the presence of lactoferrin alone (see Figs 6 and 7b). In the case of serotype O1, the generation time was increased from 24 to 43 min.

In view of this apparent lack of antibacterial activity, the high molecular weight fraction was obtained by gel filtration of the equivalent of 25 ml of milk on Bio gel A-5 M. This material contained IgA, but no detectable IgM. When mixed with lactoferrin, this fraction exerted a bacteriostatic effect against *E. coli* O111, but had no effect on *E. coli* O1 or O55 (Fig. 8). When tested at a concentration equivalent to 25% V/V whole milk with an inoculum of $1·3 \times 10^4$ *E. coli* per ml, the fraction failed to exert a bactericidal effect since the viable

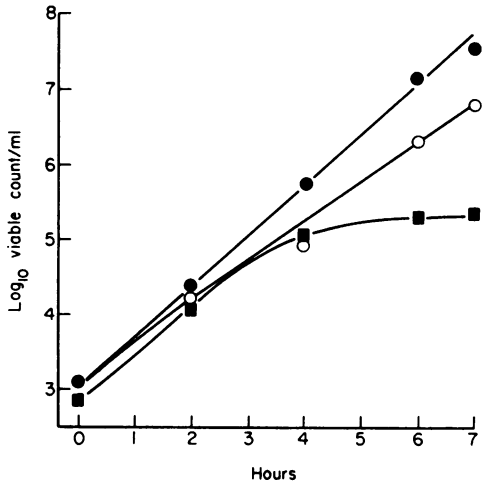

Figure 8. Effect of high molecular weight IgA fraction (1·8 mg/ml) from milk sample C on *E. coli* in the presence of lactoferrin (TIBC 20 μM): (●) serotype O1; (○) serotype O55; (■) serotype O111.

counts of the control and test sample at 2 h were $4{\cdot}4 \times 10^5$ per ml and $2{\cdot}2 \times 10^5$ per ml respectively.

Sample D. Since the whole milk failed to inhibit the growth of either *E. coli* O1 or O55 (Fig. 2), the fractions isolated from this milk were tested for antibacterial activity only against *E. coli* O111. In view of the low yields and relatively poor biological activity of some of the IgA samples isolated by a combination of gel filtration and ion exchange chromatography, it was decided to use only gel filtration for the fractionation of this sample. Five ml of clarified and 4 times concentrated milk was fractionated by gel filtration on a 3·3 × 95 cm column of Bio gel A-5 M and a 3·3 × 95 cm column of Bio gel A-0·5 M coupled in series. Fraction I eluting at 380 ml, contained 12·2 mg protein, fraction II eluting at 660 ml, contained 12·8 mg protein and fraction III eluting at 806 ml, contained 8·4 mg protein. When horse serum was separated on the same columns, the elution volume of IgM was found to be 400 ml which is quite close to the value for fraction I from the milk. Immunodiffusion tests showed that fraction I contained a trace of IgA but no IgM or IgG could be detected. Fraction II gave a strong reaction with antihuman IgA but failed to react with either anti human IgM or IgG. Fraction III gave a strong reaction with antihuman

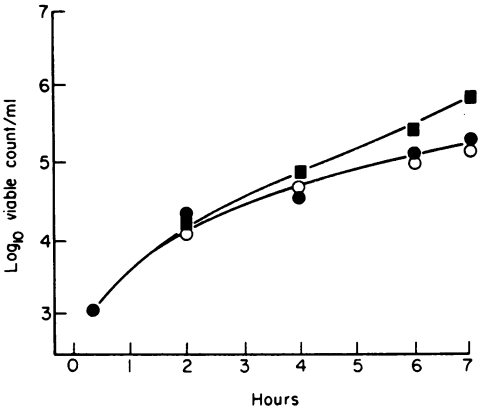

Figure 9. Effect of fractions from milk sample D in the presence of lactoferrin (TIBC 20 μM) on the growth of *E. coli* O111: (●) fraction I (1·5 mg/ml); (○) fraction II (1·3 mg/ml); (■) fraction III (1·0 mg/ml).

milk serum but failed to react with any of the specific anti Ig sera.

Fractions I and II, when tested at a concentration of 1·3 mg per ml, exerted a bacteriostatic effect on *E. coli* O111 in the presence of lactoferrin, fraction III appeared to be less active (Fig. 9). When tested at 0·16 mg per ml, all three fractions gave similar growth curves for 6 h after which the bacteria began

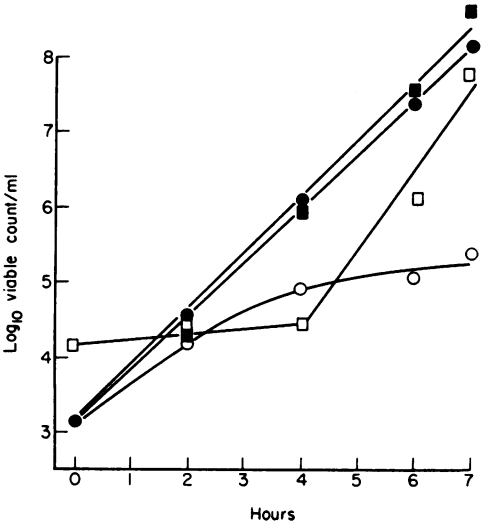

Figure 10. Effect of IgA fraction II (1·3 mg/ml) from milk sample D on *E. coli* O111: (●) IgA alone; (○) IgA plus lactoferrin (TIBC 20 μM, 17·4% saturated with Fe^{3+}); (■) IgA plus lactoferrin 200% saturated with Fe^{3+}; (□) IgA in 50% v/v foetal calf serum.

to multiply (data not shown). Further experiments with the main IgA fraction II, showed that it had little or no effect on the growth of the organism in the absence of lactoferrin and also failed to induce a bactericidal effect in the presence of foetal calf serum (Fig. 10). As was found previously with a mixture of lactoferrin and specific *E. coli* O111 antiserum (Bullen *et al.*, 1972), the bacteriostatic effect of the IgA plus lactoferrin was abolished by saturating the lactoferrin with Fe^{3+} (Fig. 10).

DISCUSSION

Human colostrum contains approximately 5 mg secretory IgA per ml whilst mature milk contains approximately 2 mg per ml (Mata & Wyatt, 1971; Michael *et al.*, 1971). Human milk has high agglutinating titres to a variety of *E. coli* serotypes (Gindrat, Gothefors, Hanson & Winberg, 1972) including O55 and O111 (Arnon, Salzberger & Olitzki, 1959). This antibody has been shown to survive passage through the intestinal tract of the infant (Kenny, Boesman & Michaels, 1967; Gindrat *et al.*, 1972). Appropriate vaccination procedures when applied to pregnant animals, give rise to O specific secretory IgA antibodies in the colostrum (Eddie, Schulkind & Robbins, 1971; Steele, Chaicumpa & Rowley, 1974). The purified IgA was highly protective against *Vibrio cholerae* in the infant mouse (Heddle & Rowley, 1975b; Steele *et al.*, 1974).

It was found that two out of three human milk samples tested were capable of inducing bacteriostasis of three potentially pathogenic serotypes of *E. coli*, namely O1, O55 and O111. The third sample was active only on O111 (Figs 1 and 2). Although of limited scope, these results are in accord with work which has demonstrated the presence, in human milk, of antibodies to a wide range of *E. coli* serotypes (Arnon *et al.*, 1959; Gindrat *et al.*, 1972). The bacteriostatic titre against *E. coli* O111 was at least 8 (Fig. 3) and was stable to pasteurization. Two out of three samples tested were active against *E. coli* O111 in the bactericidal test (Table 1) suggesting the presence of either IgM or IgG antibodies to this organism. Although the bacteriostatic effect could not be absorbed out by 1×10^{10} heat killed *E. coli* O111 per ml, it could be removed by means of the Boivin antigen suggesting that the antibody involved in the bacteriostatic effect reacts with the lipopolysaccharide of the whole cell (Fig. 4). It may be that the antibodies involved are of very low avidity.

Studies have also been made of the role of bacterial iron metabolism in infection. Experimentally, both the bacteriostatic and bactericidal effects of serum and milk (Bullen & Rogers, 1969; Bullen *et al.*, 1972) and the natural resistance of animals to *E. coli* (Bullen, Leigh & Rogers, 1968) can be abolished by iron compounds. Serum and human milk contain the iron binding proteins, transferrin and lactoferrin respectively, which have association constants for Fe^{3+} of approximately 10^{36} (Aasa, Malmstrom, Saltman & Vanngard, 1963; Aisen & Leibman, 1972). In order to obtain sufficient iron for multiplication, *E. coli* O141 was found to secrete derivatives of enterochelin (the cyclic trimer of 2,3-dihydroxybenzoyl serine) which transport Fe^{3+} from transferrin to the bacterial cell (Rogers, 1973). *E. coli* O111 also secretes enterochelin and its derivative when exposed to transferrin alone (Rogers, 1973; Rogers *et al.*, 1977). The failure of this serotype to multiply in serum was therefore ascribed to the presence of antibody which interfered with the synthesis of enterochelin by the bacterial cell. Since it is clear that enterochelin uptake is not affected by antibody (Rogers *et al.*, 1977), the fact that the addition of 0·1 μM enterochelin to milk at 5 h, also reverses bacteriostasis (Fig. 5), makes it likely that a similar series of reactions is responsible for the bacteriostatic effects of both serum and milk. In the case of immune horse serum, the antibodies involved in bacteriostasis are of the IgG class (Rogers, 1976). In view of these findings, it was decided to determine the class of antibody involved in the bacteriostatic effect of human milk.

Since IgA is the major immunoglobulin present in human colostrum and milk (Mata & Wyatt, 1971; Michael *et al.*, 1971) the main effort has been directed towards the isolation of this component. A major factor in assessing the antibacterial activity of IgA preparations is the necessity to show that they are free from both IgG and IgM (Steele *et al.*, 1974; Heddle & Rowley, 1975b). Immunodiffusion techniques can be a useful guide (Heddle & Rowley, 1975a) but when working with *E. coli* O111, the sensitive bactericidal test can be applied (Knop, Breu, Wernet & Rowley, 1971). The main IgA fraction was isolated from 4 samples of human milk by a combination of gel filtration and ion-exchange chromatography (see Results). The IgA fraction from milk A was capable of in-

ducing bacteriostasis of *E. coli* in the presence of lactoferrin (Fig. 6). No IgG or IgM could be detected in this sample. Although milk B was active against 3 *E. coli* serotypes the IgA fraction isolated from it when mixed with lactoferrin did not produce true bacteriostasis (Fig. 7a). The activity against *E. coli* O55 (Fig. 7b) was associated with a high molecular weight fraction in which the only detectable immunoglobulin was IgA. Milk C also contained a high molecular weight fraction which reacted only with antiserum to IgA and was capable of inducing bacteriostasis of *E. coli* O111 (Fig. 8). This fraction was inactive in the bactericidal test. In the case of milk D immunodiffusion tests showed that the main IgA fraction contained no detectable IgG or IgM while only IgA could be detected in the fraction corresponding to IgM in terms of molecular weight. These fractions induced bacteriostasis of *E. coli* O111 in the presence of lactoferrin but were inactive in the bactericidal test. Except for its failure to activate complement, the main IgA fractions possessed the same antibacterial properties as immune IgG. Thus the fractions induced bacteriostasis only in the presence of an iron binding protein, a process which could be reversed by saturating the iron binding capacity (Fig. 10) (Rogers, 1976).

Hence, of seven IgA-containing fractions isolated from four different samples of human milk, four have been shown to induce bacteriostasis of *E. coli* O111 in the presence of lactoferrin, whilst one sample out of three was active against *E. coli* O55. Although they did not induce true bacteriostasis, a further two out of seven samples produced significant increases in the generation time of *E. coli* O111, whilst two out of three samples produced this effect with *E. coli* O1. These results probably arise from samples taken from random subjects exposed to a variety of bacterial antigens. In addition, losses of antibacterial activity may occur during the fractionation procedures, milk B may be one such case. The exact nature of the IgA-containing, high molecular weight fraction is unknown. It could correspond to the 18S polymer described by Tomasi, Tan, Solomon & Prendergast (1965).

It is now worthwhile considering how the bacteriostatic effect of IgA and lactoferrin may contribute to the resistance of the young animal to gastrointestinal infection. The fact that secretory IgA is not synthesized by the human infant during the first 10 days of life (Haneberg & Aarskog, 1975) together with the antitryptic activity of colostrum (Laskowski & Laskowski, 1951) suggests that colostral IgA may play an important role in protecting the intestinal tract. Experimentally, it is quite clear that secretory IgA provides protection against enteric pathogens such as *V. cholerae* (Fubara & Freter, 1973) but the mechanism by which bacterial growth is suppressed (Kohler, 1974) has not been clarified. In the case of the newborn, suckled animal, it is considered that the bacteriostatic mechanism outlined above could operate in the lumen of the small intestine. The ability of haematin to enhance bacterial growth in the small intestine of suckled guinea-pigs is in accord with this idea (Bullen *et al.*, 1972). The antibody molecules involved in bacteriostasis appear to react with the lipopolysaccharide of the bacterial cell (Fig. 4) (Fitzgerald & Rogers, 1976). In the case of *V. cholerae*, the O antigen increases the protective effect of parenteral vaccines (Svennerholm & Holmgren, 1976) and can also neutralize the protective effect of secretory IgA (Steele *et al.*, 1974). These observations emphasize the importance of antibacterial immunity in resistance to enteric pathogens.

In the case of the adult animal, the possible role of bacteriostasis in protection against enteric pathogens is less obvious. IgA (Girard & de Kalbermatten, 1970) and lactoferrin (Masson, 1970) are secreted by the intestinal mucosa, but the digestive power of the chyme could well reduce their effectiveness in the lumen of the intestine. Lactoferrin can easily be detected in washings from the small intestine of the rabbit but the chyme from orally immunized animals supports rapid growth of the homologous organism (Rogers & Synge, unpublished observations). Since both lactoferrin and IgA would be at their highest concentration at the mucosal surface, the failure of bacteria to multiply close to the villi in the immune animal (Schrank & Verwey, 1976) could be explained if the IgA were assumed to exert an antiadhesive effect (Fubara & Freter, 1973) and simultaneously, together with lactoferrin, the bacteriostatic effect described above.

REFERENCES

AASA R., MALMSTROM B.G., SALTMAN P. & VANNGARD T. (1963) The specific binding of iron III and copper II to transferrin and conalbumin. *Biochim. Biphys. Acta*, **75**, 203.

AISEN P. & LEIBMAN A. (1972) Lactoferrin and transferrin: A comparative study. *Biochim. Biophys. Acta*, **257**, 314.

ARNON H., SALZBERGER M. & OLITZKI A.L. (1959) The appearance of antibacterial and antitoxic antibodies in maternal sera, umbilical cord blood and milk: Observations on the specificity of antibacterial antibodies in human sera. *Pediatrics*, **23**, 86.

BULLEN C.L. & WILLIS A.T. (1971) Resistance of the breast-fed infant to gastroenteritis. *Brit. med. J.* **iii**, 338.

BULLEN J.J., LEIGH L.C. & ROGERS H.J. (1968) The effect of iron compounds on the virulence of *Escherichia coli* for guinea-pigs. *Immunology*, **15**, 581.

BULLEN J.J. & ROGERS H.J. (1969) Bacterial iron metabolism and immunity to *Pasteurella septica* and *Escherichia coli*. *Nature* (*Lond.*), **224**, 380.

BULLEN J.J., ROGERS H.J. & LEIGH L. (1972) Iron binding proteins in milk and resistance to *Escherichia coli* infections in infants. *Brit. med. J.* **i**, 69.

EDDIE D.S., SCHULKIND M.L. & ROBBINS J.B. (1971) The isolation and biological activities of purified secretory IgA and IgG anti-*Salmonella typhimurium* O antibodies from rabbit intestinal fluid and colostrum. *J. Immunol.* **106**, 181.

EVANS D.G., SILVER R.P., EVANS D.J., CHASE D.G. & GORBACH S.L. (1975) Plasmid controlled colonisation factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. *Infect. Immun.* **12**, 656.

FITZGERALD S.P. & ROGERS H.J. (1976) Bacteriostatic effect of serum on *Escherichia coli* O111: Elucidation of the antibody binding site. *Proc. Soc. gen. Microbiol.* **3**, 110.

FUBARA E.S. & FRETER R. (1973) Protection against enteric bacterial infection by secretory IgA antibodies. *J. Immunol.* **111**, 395.

GERRARD J.W. (1974) Breast-feeding: Second thoughts. *Pediatrics*, **54**, 757.

GINDRAT J.J., GOTHEFORS L., HANSON L.A. & WINBERG J. (1972) Antibodies in human milk against serogroups most commonly found in neonatal infections. *Acta Paed. Scand.* **61**, 587.

GIRARD J.P. & DE KALBERMATTEN A. (1970) Antibody activity in human duodenal fluid. *Europ. J. clin. Invest.* **1**, 188.

HANEBERG B. & AARSKOG D. (1975) Human faecal immunoglobulins in healthy infants and children and in some with diseases affecting the intestinal tract or the immune system. *Clin. exp. Immunol.* **22**, 210.

HANSON L.A. & JOHANSSON B.G. (1962) Immunological characterisation of chromatographically separated protein fractions from human colostrum. *Int. Arch. Allergy appl. Immunol.* **20**, 65.

HEDDLE R.J. & ROWLEY D. (1975a) Dog immunoglobulins I. Immunochemical characterisation of dog serum, parotid saliva, colostrum, milk and small bowel fluid. *Immunology*, **29**, 185.

HEDDLE R.J. & ROWLEY D. (1975b) Dog immunoglobulins II. The antibacterial properties of dog IgA, IgM and IgG antibodies to *Vibrio cholerae*. *Immunology*, **29**, 197.

KENNY J.F., BOESMAN M.I. & MICHAELS R.H. (1967) Bacterial and viral coproantibodies in breast-fed infants. *Pediatrics*, **39**, 202.

KNOP J., BREU H., WERNET P. & ROWLEY D. (1971) The relative antibacterial efficiency of IgM, IgG and IgA from pig colostrum. *Austr. J. exp. Biol. med. Sci.* **49**, 495.

KOHLER E.M. (1974) Protection of pigs against neonatal colibacillosis with colostrum and milk from orally vaccinated sows. *Am. J. vet. Res.* **35**, 331.

LASKOWSKI M. & LASKOWSKI M. (1951) Crystalline trypsin inhibitor from colostrum. *J. biol. Chem.* **190**, 563.

MASSON P. (1970) *La Lactoferrine*. Editions Arscia S.A. Brussels.

MATA L.J. & URRUTIA J.J. (1971) Intestinal colonization of breast-fed children in a rural area of low socioeconomic level. *Ann. N.Y. Acad. Sci.* **176**, 93.

MATA L.J. & WYATT R.G. (1971) Host resistance to infection. *Am. J. clin. Nutr.* **24**, 976.

MICHAEL J.G., RINGENBACK R. & HOTTENSTEIN S. (1971) The antimicrobial activity of human colostral antibody in the newborn. *J. infect. Dis.* **124**, 445.

ORSKOV F. & BIERING SORENSEN K. (1975) *Escherichia coli* serogroups in breast-fed and bottle-fed infants. *Acta path. microbiol. Scand.* **83**, 25.

ROGERS H.J. (1973) Iron binding catechols and virulence in *Escherichia coli*. *Infect. Immun.* **7**, 445.

ROGERS H.J. (1976) Ferric iron and the antibacterial effects of horse 7S antibodies to *Escherichia coli* O111. *Immunology*, **30**, 425.

ROGERS H.J., SYNGE C., KIMBER B. & BAYLEY P.M. (1977) Production of enterochelin by *Escherichia coli* O111. *Biochim. Biophys. Acta*, **497**, 548.

ROWLEY D. (1968) Sensitivity of rough gram negative bacteria to the bactericidal action of serum. *J. Bacteriol.* **95**, 1647.

SARFF L.D., MCCRACKEN G.H., SCHIFFER M.S., GLODE M.P., ROBBINS J.B., ORSKOV I. & ORSKOV F. (1975) Epidemiology of *Escherichia coli* K1 in healthy and diseased newborns. *Lancet*, **i**, 1099.

SCHRANK G.D. & VERWEY W.F. (1976) Distribution of cholera organisms in experimental *Vibrio cholerae* infections: Proposed mechanisms of pathogenesis and antibacterial immunity. *Infect. Immun.* **13**, 195.

SMITH H.W. (1972) The nature of the protective effect of antisera against *Escherichia coli* diarrhoea in piglets. *J. med. Microbiol.* **5**, 345.

STAUB A.M. (1965) Bacterial lipido-proteino-polysaccharides (O somatic antigens). *Methods in Carbohydrate Chemistry*, **5**, 92.

STEELE E.J., CHAICUMPA W. & ROWLEY D. (1974) Isolation and biological properties of three classes of rabbit antibody to *Vibrio cholerae*. *J. infect. Dis.* **130**, 93.

STOLIAR O.A., PELLEY R.P., KANIECICI-GREEN E., KLAUS M.H. & CARPENTER C.C.J. (1976) Secretory IgA against enterotoxins in breast milk. *Lancet*, **i**, 1258.

SVENNERHOLM A.M. & HOLMGREN J. (1976) Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin/toxoid. *Infect. Immun.* **13**, 735.

SVIRSKY-GROSS S. (1958) Pathogenic strains of coli (O111) among prematures and the use of human milk in controlling the outbreak of diarrhoea. *Annales Paediatrici*, **190**, 109.

TASSOVATZ B. & KOTSITCH A. (1961) Le lait de femme et protection contre les infections intestinales chez le nouveane. *Annales de Pediatrie*, **8**, 285.

TOMASI T.B. & BIENENSTOCK J. (1968) Secretory immunoglobulins. *Adv. Immunol.* **9**, 29.

TOMASI T.B., TAN E.M., SOLOMON A. & PRENDERGAST R.A. (1965) Characteristics of an immune system common to certain external secretions. *J. exp. Med.* **121**, 101.